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Microassay for measurement of binding of radiolabelled ligands to cell surface molecules

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An improved technique for measuring the binding of radiolabelled ligands to cell surface molecules has been developed by modification of a procedure using centrifugation through a water-immiscible oil to separate free and cell-bound ligand. It maximises the percentage of ligand bound since cell-bound and free ligand can be separated easily and reproducibly even when very small reaction volumes are used. This permits low levels of ligand radiolabelling and relatively low numbers of cells to be used.

Key words: Cell receptor; Ig receptor; Radiolabeled ligand

Introduction

There is an ever-increasing interest in the interaction of soluble molecules with molecules of the cell surface, examples including ligand-receptor and antibody-cellular antigen interactions. One of the most effective means of following such interactions is to radiolabel the soluble molecule (termed ligand here for convenience). The free and cell-bound ligand are most usually separated by centrifugation followed by washing procedures. The latter are time consuming, introduce manipulative errors and may disturb the equilibrium between ligand and cell receptor. A more satisfactory procedure is to layer the equilibrium mixture over a water-immiscible oil and centrifuge. The cells with bound ligand form a pellet and the medium containing free ligand remains overlaying the oil. This procedure still introduces error when transferring the equilibrium mixture from the incubation vessel to the centrifuge tube and this becomes significant if one is working with small volumes. Indeed to minimise cell numbers needed in a binding assay whilst maintaining a reasonable percentage of ligand bound it is highly desirable to work with small volumes. The alternative to this, adopted by many workers, is to accept a very low percentage of ligand bound but very heavily radiolabel the ligand so that these small percentages (often $< 1\%$) can be measured. Extensive radiolabelling may not always be desirable.

Here we describe a microassay technique for the reliable and quantitative measurement of the binding of radiolabelled ligands to cell receptors. The method is a modification of the standard procedure using immiscible oils in which the oil is layered onto the equilibrium mixture in the incubation vessel followed by centrifugation. The method typically requires only of the order of 10^6 cells per binding point to give high (25–50%) ligand binding depending on binding constant, number of receptors per cell and ligand concentration. Alternatively the method can be used with many fewer cells and a consequent fall in percentage ligand binding.

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Experimental

Method

Cells were incubated to equilibrium with radiolabelled ligand in microfuge tubes (400 μl polyethylene, diameter 5 mm, Alpha Laboratories) which had been pre-cut to 2.5 cm length to permit access with automatic pipette tips. The incubation medium, total volume 20 μl, contained 0.2% BSA to minimise non-specific binding of ligand to the plastic tubes. For studying IgG binding to the high affinity Fc receptor (FcRI) on human monocytes ($K \approx 5 \times 10^8$ M^{-1}, number of receptors/cell $\approx 1 \times 10^4$) we typically used 10^6 cells per tube with an ^{125}I-labelled IgG concentration of 1–4 nM and incubated for 1 h at 37°C.

The mixture was then overlayed with 50 μl of Versilube F50 oil (Alfa Chemicals Ltd., Woringham) and micro-centrifuged at $11000 \times g$ for 5 min. Medium containing free ligand passed upward through the oil whilst the cells with bound ligand pelleted beneath it. Each tube was then held horizontally over the rim of a counting tube and a scalpel blade used to cut through the lower part of the oil layer directly above the tube rim. The tip containing the cell pellet fell into the counting tube and the remainder of the microfuge tube containing the medium and some oil was quickly placed in a second counting tube positioned alongside. In our experience the oil did not contain significant levels of radioactivity and so its distribution between the two counting tubes was unimportant.

The choice of microfuge tube diameter was found to be critical since with wider tubes surface tension effects can lead to problems in separating medium and oil layers.

Results and discussion

The method described was successfully applied to the study of IgG binding to FcRI on human monocytes and the monocyte-like cell line, U937 (Woof et al., 1984; Leatherbarrow et al., 1985; Partridge et al., 1986; Woof et al., 1986). Scatchard analysis gave a binding constant for pooled human IgG of $2.44 \pm 0.36 \times 10^8$ M^{-1} (mean ± SEM) which compares closely with that obtained by washing methods (Alexander et al., 1978) or by layering over oil (Fries et al., 1982; Kurlander and Batker, 1982).

We found the experimental data to be generally more reproducible with the method described than the previous oil method. For each method a typical sample of 50 duplicate readings were analysed statistically. The values for the standard deviation of each set of duplicates from their mean were collated and averaged. Thus, the mean of standard deviations of the duplicates from their mean for the previous oil method was found to be 1.017 compared with the much lower figure of 0.375 for the method described here. This is as expected in view of the reduced manipulation required.

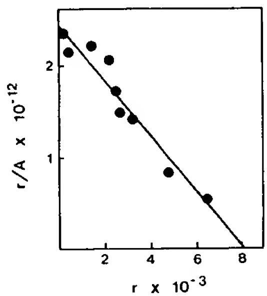

Fig. 1. Scatchard plot of ^{125}I-labelled pooled human IgG binding to U937 cells. The binding assay was carried out at several different concentrations of ^{125}I-IgG ranging from 1.23 $\times 10^{-8}$ to 6.15×10^{-11} M. At each concentration tested duplicate incubation tubes containing 8.5×10^5 U937 cells and the ^{125}I-IgG were used. One contained excess (1×10^{-6} M) unlabelled monomeric human IgG to give a value for non-specific binding while the other tube gave the value for total binding. After incubation the free and bound ^{125}I-IgG were separated and total and non-specific binding calculated for each ^{125}I-IgG concentration. Non-specific binding was subtracted from total binding to give the specific binding values used in the calculations. The number of moles of ^{125}I-IgG bound per mole of cells, r, was calculated using the following relationship:

$$r = \frac{6 \times 10^{23} \times IgG_b}{\text{number of cells/l}}$$

where IgG_b is the concentration of bound ^{125}I-IgG. A represents the concentration of free ^{125}I-IgG. The slope of the plot indicates a K_{ass} of 3.0×10^8 M^{-1}. The abscissa intercept gives a value of 8×10^3 receptors/cell.

A typical Scatchard plot using this modified method is shown in Fig. 1. In four Scatchard plots least-squares analysis gave coefficients of correlation (r) in the range 0.90–0.98 with a mean value of 0.95 ± 0.02 ($\pm$SEM) and abscissa intercept (receptor number/cell) values in the range $5.5–8.01 \times 10^3$ with a mean of $7.5 \pm 0.68 \times 10^3$ ($\pm$SEM). These estimations were made on cells grown at relatively high cell concentrations ($\sim 0.9 \times 10^6$/ml). The lower numbers of FcRI per cell than those previously reported (Anderson and Abraham, 1980; Raychaudhuri et al., 1985) is probably due to an effect of high cell growth concentrations on FcRI expression. We regularly obtain $> 25\%$ ligand binding by this procedure and this has permitted us to easily and reliably investigate the effects of inhibitors on the IgG-monocyte interaction (e.g., Woof et al., 1984, 1986; Partridge et al., 1986).

Since the binding constant of monoclonal antibodies to cellular antigens is typically of the order of 10^8 M^{-1} and the number of antigens per cell $\geqslant 2 \times 10^4$, the method could find general applicability in studies of direct antibody binding to cells.

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